NADPH-dependent extracellular superoxide production is vital to photophysiology in the marine diatom *Thalassiosira oceanica*

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Reactive oxygen species (ROS) like superoxide drive rapid transformations of carbon and metals in aquatic systems and play dynamic roles in biological health, signaling, and defense across a diversity of cell types. In phytoplankton, however, the ecophysiological role(s) of extracellular superoxide production has remained elusive. Here, the mechanism and function of extracellular superoxide production by the marine diatom *Thalassiosira oceanica* are described. Extracellular superoxide production in *T. oceanica* exudates was coupled to the oxidation of NADPH. A putative NADPH-oxidizing flavoenzyme with predicted transmembrane domains and high sequence similarity to glutathione reductase (GR) was implicated in this process. GR was also linked to extracellular superoxide production by whole cells via quenching by the flavoenzyme inhibitor diphenylene iodonium (DPI) and oxidized glutathione, the preferred electron acceptor of GR. Extracellular superoxide production followed a typical photosynthesis-irradiance curve and increased by 30% above the saturation irradiance of photosynthesis, while DPI significantly impaired the efficiency of photosystem II under a wide range of light levels. Together, these results suggest that extracellular superoxide production is a byproduct of a transplasma membrane electron transport system that serves to balance the cellular redox state through the recycling of photosynthetic NADPH. This photoprotective function may be widespread, consistent with the presence of putative homologs to *T. oceanica* GR in other representative marine phytoplankton and ocean metagenomes. Given predicted climate-driven shifts in global surface ocean light regimes and phytoplankton community-level photoacclimation, these results provide implications for future ocean redox balance, ecological functioning, and coupled biogeochemical transformations of carbon and metals.

reactive oxygen species | photosynthesis | oxidative stress | biogeochemistry

All aerobic organisms generate reactive oxygen species (ROS). The accelerated production of ROS under adverse conditions, or oxidative stress, has toxic effects on cellular viability and health (1). However, awareness is increasing that the biological production of ROS also drives vital processes in organisms spanning bacteria to mammals, such as redox sensing and homeostasis (2, 3), growth (2, 4–7), innate immunity (8, 9), and carbon acquisition (10).

Biological ROS production often begins with the single-electron reduction of O_2 to superoxide ($O_2^{\bullet-}/HO_2^{\bullet}$). In a typical eukaryotic phytoplankton cell, superoxide is generated intracellularly within chloroplasts, mitochondria, and peroxisomes, as well as extracellularly at the cell surface and within cell-free exudates (11). The ability to produce extracellular superoxide is taxonomically widespread among phytoplankton (11, 12), but its potential physiological role remains unknown (11). For example, phytoplankton communities are major contributors to natural levels of superoxide within aquatic environments (13), where ROS shape biological interactions over broad trophic scales (11) and control the cycling of carbon, as well as nutrient and toxic metals (13). However, this biogeochemistry is not necessarily related to the potential biological function of extracellular superoxide production. Indeed, superoxide can directly alter the speciation and bioavailability of dissolved iron (14), a micronutrient that often limits photosynthetic activity in the oceans, but evidence for the regulation of extracellular superoxide production by phytoplankton in response to iron availability is not consistent (14–17).

Phytoplankton produce extracellular superoxide under ideal growth conditions within culture incubations (11). In fact, production rates of extracellular superoxide are commonly highest during periods of active growth and typically do not increase in stationary phase when cells become stressed by resource limitation (11). Thus, rather than serving strictly in stress response, extracellular superoxide production may play a role in the vitality and fundamental physiology of phytoplankton cells. For example, extracellular superoxide production by many phytoplankton is stimulated with light exposure, indicating a link to photosynthesis (11). While this relationship could suggest leakage of intracellular superoxide, extracellular superoxide is unlikely to originate directly from chloroplasts, as superoxide cannot readily diffuse

Significance

Superoxide and other reactive oxygen species (ROS) are commonly regarded as harmful progenitors of biological stress and death, but this view has been changing. Indeed, many phytoplankton actively generate extracellular superoxide under ideal growth conditions for reasons that are mysterious. Results from this study suggest that extracellular superoxide production by the marine diatom *Thalassiosira oceanica* may promote photosynthetic health by modulating the oxidation state of the cellular $NADP^+$/NADPH pool. The key enzyme implicated in this process is present in other representative marine phytoplankton and global ocean metagenomes. Overall, these findings transform the perceived role of superoxide in the health and functioning of phytoplankton and present implications for redox balance, biogeochemistry, and ecology in the future ocean.

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across biological membranes due to the dominance of the negatively charged species at typical physiological pH (18), its brief intracellular lifetime (~μs), and short diffusive distance (~100's of nm) (1). Alternatively, as in higher eukaryotes (19), extracellular superoxide production by some phytoplankton species is mediated by cell surface NADPH-oxidizing enzymes such as NADPH oxidase (Nox) (20–22). Photosynthesis may therefore control extracellular superoxide production indirectly by supplying NADPH to such enzymes (23–25).

Maintaining redox balance of the NADPH/$NADP^+$ pool is an important aspect of photosynthetic metabolism. In particular, the regeneration of $NADP^+$, the terminal electron acceptor of the light reactions, is necessary to furnish the ideal NADPH:ATP ratio required for carbon fixation and to achieve the optimal redox state of the photosynthetic electron transport chain (26). Whether the NADPH dependence of extracellular superoxide production is related to its physiological function has not been explored, however. Here, we evaluated the mechanism and function of extracellular superoxide production in the model marine diatom *Thalassiosira oceanica* CCMP1005. Our results support the hypothesis that NADPH-dependent production of extracellular superoxide plays an essential role in phytoplankton photophysiology.

Results and Discussion

Batch cultures of *T. oceanica* were grown with replete initial nutrient concentrations under optimal light conditions, and whole cells were analyzed for net extracellular superoxide production by a flow-through chemiluminescence approach described previously (5, 24, 27). Results revealed that extracellular superoxide production rates were highest in exponential growth phase (*SI Appendix*, Fig. S1 and *Results and Discussion*; $P < 0.05$, Tukey honestly significance difference [HSD]). As observed in other phytoplankton (11), this decline of net extracellular superoxide production with increasing age challenges the perception of superoxide as a stress-response molecule, given the continual drawdown of nutrients and the accumulation of cellular stress that occurs over time in batch cultures. These results therefore suggest that extracellular superoxide production may be a constitutive, or even beneficial physiological process in *T. oceanica*.

Extracellular Superoxide Production Depends on the Oxidation of NADPH. To investigate the mechanism of extracellular superoxide production by *T. oceanica*, we examined protein samples concentrated from the cell exudate of midexponential phase batch cultures (*SI Appendix*, Fig. S2). The addition of NADPH stimulated superoxide production by these concentrated exoprotein samples, which was inhibited by protein denaturation via boiling, ruling out nonenzymatic sources (Fig. 1*A*). Superoxide production by concentrated exoproteins was also quenched by diphenylene iodonium (DPI), a broad-spectrum inhibitor of Nox and other flavoenzymes (Fig. 1*A*). To identify the extracellular superoxide-generating enzyme present in *T. oceanica* exudates, concentrated extracellular proteins were separated by native polyacrylamide gel electrophoresis (PAGE) and then analyzed with an in-gel activity assay for superoxide production using the superoxide probe nitroblue tetrazolium (NBT) (28, 29). Activity gels indicated the production of superoxide in a single NBT stained band of ~100 kDa, which was reproducible across several biological replicates (Fig. 1 *B–D*).

A diversity of proteins was present in the NBT activity band, as revealed by peptide fingerprinting via liquid chromatography and tandem mass spectrometry (LC/MS/MS) (Dataset S1 and *SI Appendix*, Figs. S3 and S4). Nox homologs were not detected in the NBT band, despite several putative Nox homologs encoded within the *T. oceanica* genome (*SI Appendix*, Fig. S5 and Table S1). Instead, results revealed only one protein that was consistent with the enzyme

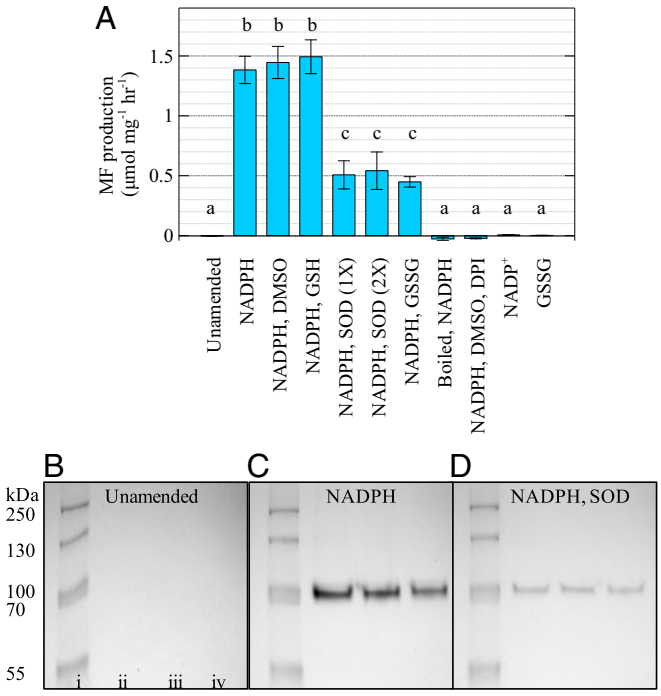

Fig. 1. Superoxide production by *T. oceanica* concentrated extracellular proteins in the presence of inhibitors and stimulants. (*A*) NBT reduction to monoformazan (MF) is driven by superoxide production by bulk concentrated extracellular proteins (average ± SD of 3 biological replicates). SOD treatments show some superoxide-independent NBT reduction, which is eliminated by boiling or incubation with DPI. Superoxide production is therefore represented after accounting for SOD controls and applying the MF:superoxide reaction stoichiometry (1:2) (28). Treatments not connected by the same letter are significantly different (Tukey HSD, $P < 0.01$). (*B–D*) Activity gels reflecting superoxide production by concentrated extracellular proteins separated by native PAGE and incubated in 5 mM Tris (pH = 8) with (*B*) no amendment (*C*) NADPH, or (*D*) NADPH and SOD. Lane i is a prestained protein ladder, and each subsequent lane (ii–iv) represents a biological replicate.

activity results: an oxidoreductase with binding sites for NADPH and a flavin prosthetic group (*SI Appendix*, Fig. S4). Based on sequence analysis, this enzyme has high similarity to glutathione reductase (GR) (*SI Appendix*, Figs. S6 and S7, Tables S2–S5, and *Results and Discussion*). In fact, the results could not distinguish between 2 putative isoforms of GR in the *T. oceanica* genome, which are referred to here as *T. oceanica* GR1 (NCBI accession EJK45974) and GR2 (EJK71311), or simply ToGR1 and ToGR2, respectively.

Glutathione reductases are homodimeric proteins composed of ~55-kDa monomers (30), so the presence of ToGR1 and ToGR2 in the ~100-kDa activity band (Fig. 1 *B–D*) is consistent with the expected molecular weight of an active GR homodimer. While protein purification is required before extracellular superoxide production can be unequivocally attributed to *T. oceanica* GR, NADPH-dependent superoxide production by GR and related flavoenzymes from yeast, plant, and bovine has been reported previously (31–33). Furthermore, the ability of GR from *Saccharomyces cerevisiae* (ScGR) to produce superoxide in vitro in the presence of NADPH was confirmed here (*SI Appendix*, Fig. S8). In a prior study, the inability of formaldehyde additions to eliminate extracellular superoxide production by *T. oceanica* was interpreted to indicate that a passive photochemical process contributes to the generation of extracellular superoxide by this species and related diatoms (24). However, we demonstrate here that formaldehyde not only failed to inhibit NADPH-dependent superoxide production by ScGR, it actually stimulated superoxide production (*SI Appendix*, Fig. S8). Therefore,

the lack of formaldehyde inhibition of extracellular superoxide production by *T. oceanica* (24) cannot necessarily rule out enzymatic mechanisms such as the potential involvement of GR.

GR typically catalyzes the NADPH-dependent reduction of glutathione disulfide (GSSG) to 2 molecules of reduced glutathione (GSH). In turn, GSH is a critical antioxidant, which can degrade hydrogen peroxide in a reaction catalyzed by the enzyme glutathione peroxidase. Thus, GR evidently oscillates between ROS-producing and ROS-degrading activities. Peroxidase enzymes responsible for extracellular superoxide production in bacteria and plants also operate via similar oscillatory redox behavior (29). In the case of *T. oceanica* GR, this catalytic versatility may potentially be regulated by the availability of the preferred electron acceptor, GSSG. For example, where GSSG levels are low, as in surrounding seawater, O_2 may substitute as the electron acceptor, thereby favoring the production of extracellular superoxide by GR. But when present, GSSG may outcompete O_2 for the enzyme's active site. Consistent with this hypothesis, additions of exogenous GSSG quenched NADPH-dependent superoxide production by concentrated extracellular proteins just as efficiently as superoxide dismutase (SOD), the enzyme that specifically degrades superoxide (Fig. 1*A*). This inhibition could not be explained by direct reaction between superoxide and GSSG or any GSH produced (Fig. 1*A*).

If GR is involved in the production of extracellular superoxide by *T. oceanica*, the enzyme would likely have to be localized on or near the cell surface, due to the limited ability of intracellular superoxide to traverse biological membranes. Indeed, sequence analysis suggests that ToGR1 and ToGR2 possess a significant probability for extracellular secretion, which was not seen in results from known intracellular GRs of human, *Saccharomyces cerevisiae*, and bacteria (*SI Appendix*, Table S6 and *Results and Discussion*). Compared with these reference GRs, ToGR1 and ToGR2 also possess unique N-terminal domains of more than 130 amino acids (*SI Appendix*, Fig. S6), which contain predicted transmembrane (TM) binding sites and/or signal peptide regions (*SI Appendix*, Figs. S6 and S9 and Tables S7 and S8), similar to the topology of transmembrane Nox enzymes (19). Consistent with these predicted TM/signal peptide domains and the presence of *T. oceanica* GR within the cell-free filtrate, we speculate that these enzymes may be reversibly bound to the plasma membrane, similar to detachable oxidoreductases that generate extracellular superoxide on the cell surface and in the exudates of other microorganisms (20, 21, 29). One mechanism of detachment likely involves the cleavage of the putative N-terminal TM binding domain. For example, based on the amino acid sequences alone, the predicted molecular weights of homodimeric ToGR1 and ToGR2 are ~140 kDa with the N-terminal domains and ~107 kDa without. Cleavage of the N-terminal domains is therefore consistent with the appearance of the functionally active protein in the ~100-kDa fraction of the cell exudate (Fig. 1 *B–D* and *SI Appendix*, *Results and Discussion*). Due to the presumed scarcity of NADPH in the aqueous environment, however, GR probably would not generate superoxide once detached, unless NADPH is provided exogenously (Fig. 1). In this way, the cleavage of GR from the membrane-binding N terminus could possibly be one mechanism for downregulating extracellular superoxide production in aging cultures of *T. oceanica* (*SI Appendix*, Fig. S1).

In agreement with the potential surface localization of the *T. oceanica* GR, extracellular superoxide production by whole cells was completely (Fig. 2*A*) and instantaneously (Fig. 2*B*) eliminated by the addition of GSSG. Due to the unlikelihood that superoxide can passively diffuse out of the cell, this result cannot be explained by the possible uptake of GSSG and reaction with cytoplasmic GR, thus underscoring the potential extracellular localization of the enzyme. Nor could this result be explained by

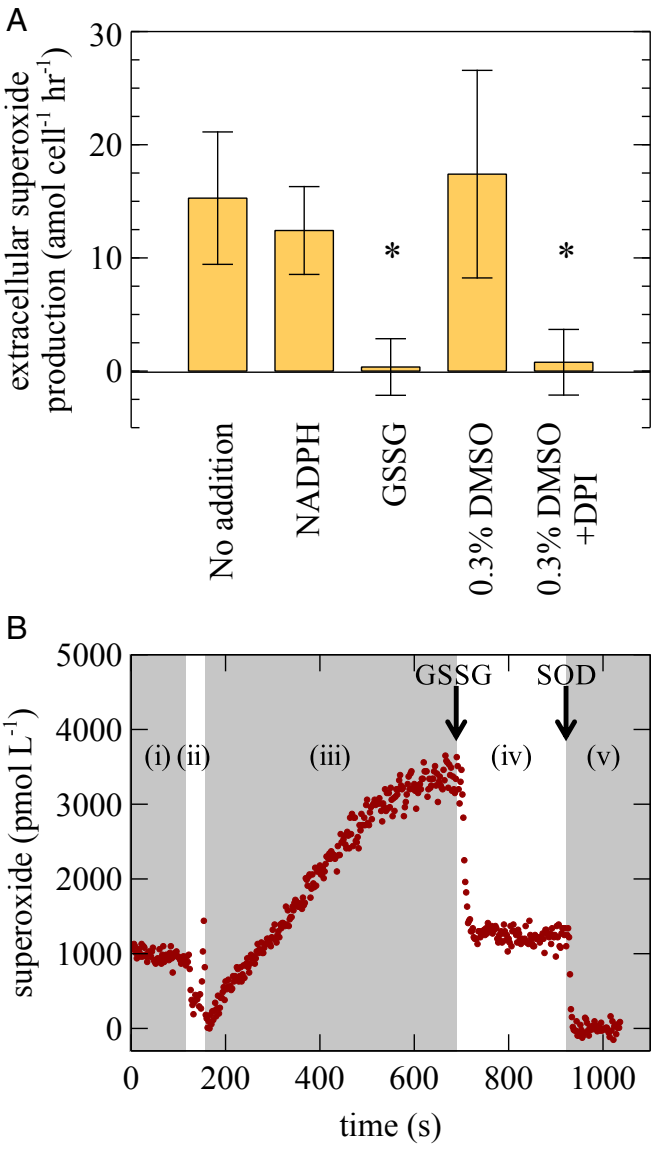

Fig. 2. Extracellular superoxide production by *T. oceanica* cells in the presence of inhibitors and stimulants. (*A*) Rates of net extracellular superoxide production, average ± SD of 4 (control, NADPH) or 3 biological replicates (all other treatments). Treatments that are significantly different from the "no addition" control are indicated by an asterisk (Dunnett test, $P < 0.05$). (*B*) Time course of extracellular superoxide concentrations reflecting the effect of GSSG in a typical FeLume run. Shaded regions correspond to (i) baseline signal of MCLA reagent + carrier solution with clean syringe filter inline, (ii) pump stopped while cells are loaded onto the filter, (iii) stabilization of cell-derived superoxide signal, (iv) superoxide levels with the addition of GSSG, and (v) SOD. Arrows indicate the point when inhibitors were added.

direct reaction between superoxide and GSSG or any GSH generated through GSSG reduction (*SI Appendix*, *Results and Discussion*). For example, superoxide calibration curves were unaffected by GSSG (*SI Appendix*, Fig. S10). In addition to GSSG, DPI also quenched extracellular superoxide production by whole cells (Fig. 2*A*), consistent with results from concentrated *T. oceanica* proteins (Fig. 1*A*), which further indicates the involvement of a cell surface flavoenzyme such as GR.

Exogenous NADPH did not stimulate extracellular superoxide production by whole cells (Fig. 2*A*), in contrast to the stimulatory effect in protein extracts (Fig. 1*A*). This result suggests that, unlike the electron acceptor binding site, which is probably

extracellular, the NADPH-binding site of the superoxide-generating enzyme may be on the intracellular side of the membrane and therefore inaccessible to extracellular NADPH. This orientation is similar to Nox (19, 34, 35) and a membrane-bound bacterial NADH dehydrogenase (36), in which the oxidation of NAD(P)H on the cytosolic side of the membrane is coupled to the direct reduction of oxygen to superoxide on the opposite side (extracellular or periplasmic, respectively). The inability of exogenous NADPH to stimulate extracellular superoxide production by *T. oceanica* therefore illustrates the essential involvement of the cytosolic NADPH pool, consistent with transplasma membrane electron transport.

Photophysiological Role of NADPH-Dependent Extracellular Superoxide Production. Based on the decoupling of extracellular superoxide production from the accumulation of cellular stress in batch cultures (*SI Appendix*, Fig. S1) and the previous finding that light exposure enhances extracellular superoxide production by *T. oceanica* (24), we investigated whether extracellular superoxide production may serve a role in photophysiology. First, we examined the effect of light across a wide range of irradiances (3 to 2,250 μmol photons m^{-2} s^{-1}). Extracellular superoxide production by *T. oceanica* responded to increasing light levels in a pattern that was consistent with a typical photosynthesis-irradiance (PI) curve, including a linear relationship at low light, followed by light saturation, and ultimately photoinhibition at high light levels (Fig. 3*A*). Like many phytoplankton (11, 23, 37), *T. oceanica* generates a baseline level of extracellular superoxide in the dark, suggesting that some extracellular superoxide production is independent from active photophysiology (23). However, net extracellular superoxide production rates were fit well by a double exponential PI model adapted from Platt et al. (38) to include dark production of extracellular superoxide ($R^2 > 0.98$ for each of 3 biological replicates; $R^2 = 0.77$ for compiled dataset; *SI Appendix*, Table S9). These results suggest strong regulation of extracellular superoxide production by photosynthetic activity, potentially via photosynthetic NADPH as a substrate for a cell surface oxidoreductase such as GR.

Irradiance experiments also revealed that extracellular superoxide production increased by ∼30% at light levels above the saturation threshold of photosynthesis (E_k) for *T. oceanica* (400 μmol photons m^{-2} s^{-1}; $P = 0.01$, 2-sample t test) (39) (Fig. 3*B*). This result is consistent with the up-regulation of extracellular superoxide production as a means to reoxidize surplus NADPH produced under saturating light levels, thereby promoting redox homeostasis via the dissipation of excess excitation energy. In this way, extracellular superoxide production may be involved in the photoacclimation response of *T. oceanica* to high light stress.

Extracellular superoxide production may serve a photoprotective role under nonstressful conditions, as well. Indeed, the NADPH:ATP stoichiometry produced in the light reactions exceeds the requirements for carbon fixation, even under optimal growth irradiance (26), so photosynthetic cells routinely employ additional electron sinks to achieve cellular redox balance. To test the effect of extracellular superoxide production on photophysiology, the efficiency of electron transport through photosystem II (PSII) was examined under a range of light levels in the presence of superoxide inhibitors. The efficiency of PSII reflects the amount of light energy used in photosynthesis as a proportion of the total amount of light absorbed. PSII efficiencies are therefore directly related to photosynthetic health. Results revealed that the flavoenzyme and extracellular superoxide production inhibitor DPI impaired the efficiency of PSII with increasing effectiveness as light intensity rose from dark (0 μmol photons m^{-2} s^{-1}), to ambient (∼10 μmol photons m^{-2} s^{-1}) and high light levels (2,250 μmol photons m^{-2} s^{-1}; Fig. 3*C*). These results suggest that extracellular superoxide production plays a photoprotective role across this wide range of irradiance, which

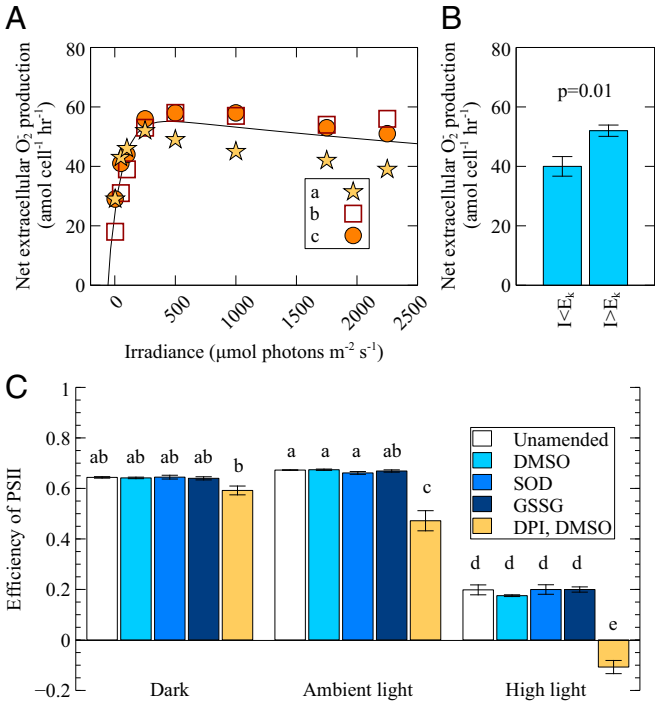

Fig. 3. Light dependence of extracellular superoxide production by *T. oceanica* cells and impacts on photosynthetic health. (*A*) Net rates of extracellular superoxide production by cells as a function of irradiance in 3 biological replicates. Data were fit using a double exponential model modified from Platt et al. (38). Model results are presented in *SI Appendix*, Table S9. (*B*) Net rates of extracellular superoxide production in *A* were binned and averaged according to irradiance (I) levels above or below the minimum saturation (E_k) of photosynthesis, 400 μmol photons m^{-2} s^{-1} (39). Error bars represent the SE of 12 observations. Results were compared using a 2-sample t test. (*C*) The average efficiency of PSII in *T. oceanica* cells exposed to chemical inhibitors of extracellular superoxide production under a range of light levels (μmol photons m^{-2} s^{-1}): dark (0), ambient light (∼10), and high light (2,250). The efficiency of PSII is a ratio that reflects the amount of light energy used in photosynthesis relative to the total amount of light absorbed. The negative value for the DPI treatment under high light reflects a lack of chlorophyll fluorescence due to damage of the photosynthetic apparatus (*SI Appendix, Supplemental Methods*). Error bars represent the SD of 3 biological replicates. Treatments not connected by the same letter are significantly different (Tukey HSD, $P < 0.05$).

is especially important under light stress. In contrast, the direct degradation of superoxide via SOD did not alter photosynthetic efficiency, indicating that extracellular superoxide, once formed, can safely be degraded without compromising the proposed redox-balancing purpose of its formation. Indeed, the removal of extracellular superoxide from *T. oceanica* cultures via daily additions of SOD not only failed to have a negative impact but actually enhanced growth rates and final cell yields ($P < 0.05$; Tukey HSD; *SI Appendix*, Fig. S11), consistent with the detoxification of this potentially reactive waste product (*SI Appendix*, *Results and Discussion*).

Unlike DPI, the extracellular superoxide production inhibitor GSSG did not diminish photosynthetic health (Fig. 3*C*). This result is expected because DPI irreversibly inhibits the activity of flavoenzymes like GR via covalent bonding (40), while GSSG most likely inhibits extracellular superoxide production by outcompeting O_2 as an electron acceptor. In this case, GSSG would not interfere with photosynthetic efficiency by halting the extracellular transport of electrons by GR. In other words, GR would still oxidize NADPH in the presence of GSSG, and a reduced waste product would form, thereby preserving photosynthetic health, but the waste product

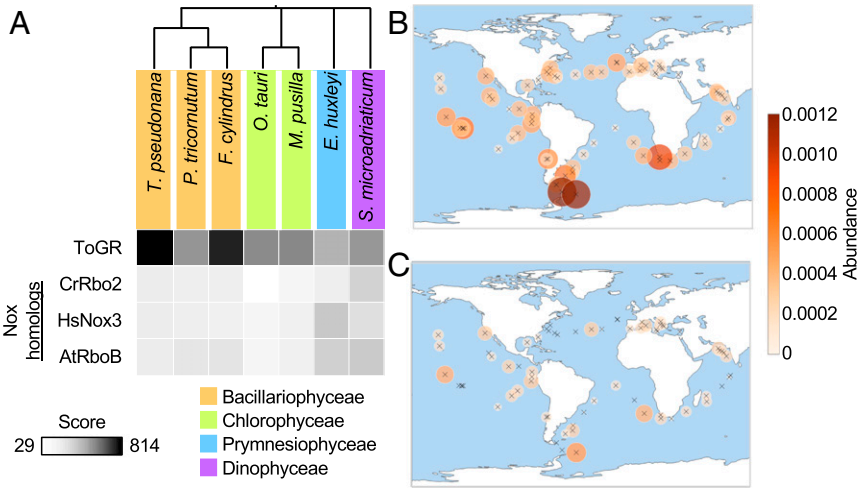

Fig. 4. Distribution of putative homologs of *T. oceanica* GR in representative phytoplankton genomes and the global ocean. (*A*) Genomes of the marine eukaryotic phytoplankton species *Thalassiosira pseudonana*, *Phaeodactylum tricornutum*, *Fragilariopsis clyindrus*, *Ostreococcus tauri*, *Micromonas pusilla*, *Emiliania huxleyi*, and *Symbiodinium microadriaticum* were searched for putative homologs to ToGR1, canonical NADPH oxidase from human (HsNox3), and respiratory burst oxidases from *C. reinhardtii* (CrRbo2) and *Arabidopsis thaliana* (AtRboB). The overall score for the top hit in each search is shown. Full results are presented in *SI Appendix*, Table S10. Relative abundance (normalized to total sequences) of putative ToGR1 homologs among eukaryotic phytoplankton sequences from (*B*) the surface ocean and (*C*) deep chlorophyll maximum. Stations are shown with an X. Size and color of circles indicate the relative abundance of phytoplankton hits with high sequence similarity to ToGR1.

would be GSH, not superoxide. Together, these findings suggest that extracellular superoxide production is a byproduct of a transplasma membrane electron transport system that serves to balance the cellular redox state and maintain proper photosynthetic electron flow under a range of physiological light levels by oxidizing NADPH, consistent with the production of extracellular superoxide by *T. oceanica* at a range of irradiances (Fig. 3*A*).

Diatom cells employ a diversity of mechanisms for alternative electron transport to maintain photosynthetic and cellular redox balance. These mechanisms do not always involve the oxidation of NADPH or the formation of superoxide, as in the case of as cyclic electron flow around PSI (41). However, similar to extracellular superoxide production, a number of alternative photosynthetic electron sinks do involve the partial or complete reduction of oxygen (*SI Appendix*, *Results and Discussion*), such as the Mehler reaction, which generates superoxide within chloroplasts (26). In contrast to such mechanisms that occur intrinsically within chloroplasts, however, extracellular superoxide production requires the export of photosynthetic reducing power from its site of formation. Excess reducing equivalents are in fact exported from chloroplasts through mechanisms that do not involve superoxide production, such as the malate valve and triosephosphate/3-phosphoglyceric acid shuttle (26, 42). Although the exact mechanisms by which NADPH may be exported from chloroplasts to fuel extracellular superoxide production are not clear, potential pathways may involve transport proteins and/or physical channels such as stromules, which connect plastids to organelles and the plasma membrane for the exchange of redox metabolites within plant cells (43). Future work should consider the quantitative contribution of extracellular superoxide production to overall pathways of alternative electron transport in phytoplankton cells, especially compared with well-known processes, such as cyclic electron flow and the Mehler reaction.

The intracellular electron sinks discussed above not only serve to dissipate excess reducing power, but also participate in redox sensing and signaling processes. For example, ROS-mediated cross-talk among chloroplasts, mitochondria, and the nucleus regulates gene expression in plants and the green alga *Chlamydomonas reinhardtii* (3, 44). Extracellular superoxide may also be involved in the transmission of redox signals from the cell surface to other organelles. For example, superoxide could be directly perceived by proteins, lipids, and other cell surface receptors that participate in signaling (3, 35, 45, 46). Alternatively, in some organisms, the dismutation of extracellular superoxide by cell surface SODs generates extracellular hydrogen peroxide, which diffuses back into the cell to elicit gene expression (3), morphogenesis (47), and proliferation (48, 49). Although speculative, the mechanism(s) by which the application of SOD stimulated growth by *T. oceanica* (*SI Appendix*, Fig. S11) may involve the induction of similar growth-promoting redox signaling pathways through the accelerated production of hydrogen peroxide (35). Furthermore, extracellular redox sensing and signaling may be necessary in the light and in the dark, which could possibly help explain the function of dark extracellular superoxide production in *T. oceanica*, highlighting a diversity of potential physiological roles for superoxide, which is also evident in other organisms.

Conclusions and Broader Significance. Overall, results from *T. oceanica* suggest that the production of extracellular superoxide, possibly by a putative cell surface flavoenzyme with similarities to GR, is constitutive, NADPH dependent, and photosynthetically regulated. Given the energetic investment necessary in this process, the stimulation of extracellular superoxide production observed under light stress, and the impairment of photosynthetic health under a range of light levels by the flavoenzyme and extracellular superoxide production inhibitor DPI, we suggest that extracellular superoxide is produced as the byproduct of a vital transplasma membrane electron transport system that promotes cellular redox homeostasis through the oxidation of photosynthetic NADPH. This potential role in photophysiology and photoacclimation may extend beyond *T. oceanica*. For example, light exposure stimulates extracellular superoxide production by many phytoplankton species (5, 11, 24), and NADPH-oxidizing enzymes have previously been implicated in extracellular superoxide production by *Thalassiosira weissflogii* (16) and *Symbiodinium* spp (23). In fact, searches of publicly available marine phytoplankton genomes revealed that putative homologs to *T. oceanica* GR are taxonomically widespread (Fig. 4*A* and *SI Appendix*, Table S10), including sequences with conserved catalytic, putative membrane binding, and/or signal peptide domains, as well as significant probabilities for extracellular secretion (*SI Appendix*, Table S11). By comparison, a similar analysis revealed weaker evidence for potential Nox homologs in these microorganisms (Fig. 4*A* and *SI Appendix*, Table S10).

Highly similar sequences to *T. oceanica* GR are also present among eukaryotic phytoplankton in the global surface ocean, as shown by an analysis of metagenomic data from the *Tara* Oceans expedition (Fig. 4 *B* and *C*). These results suggest that the potential mechanism and photoprotective role of extracellular superoxide production observed in *T. oceanica* may be present in globally relevant marine photoautotrophs. If true, this finding would have implications for the redox state of the ocean under future climate scenarios. For example, ocean warming is expected to change surface mixed layer depths, which will alter the level of light exposure that phytoplankton communities experience over

the photoperiod (50). Part of the photoacclimation response to these shifting light conditions involves large changes in phytoplankton community-level chlorophyll content over vast regions of the global surface ocean (50). Based on results from this study, similar quantitative shifts in extracellular superoxide production by phytoplankton communities should also be expected as part of this photoacclimation response, which would have profound, yet complex effects on marine ecology and biogeochemistry.

Methods

T. oceanica CCMP1005 was obtained from the National Center for Marine Algae and Microbiota (NCMA), Bigelow Laboratories, East Boothbay, Maine. Photophysiology measurements were conducted using a fluorescence induction and relaxation (FIRe) fluorometer (Satlantic). Extracellular superoxide production by whole cells was quantified with a flow-through analytical system (FeLume, Waterville Analytical) via reaction with the chemiluminescent probe methyl *Cypridina* luciferin analog (MCLA), as previously described (5, 24, 27) (*SI Appendix*, Fig. S12). Superoxide production by enzymes was quantified using a microplate assay and visualized in gel (29) based on the reaction between superoxide and the chromogenic probe NBT (28). Tryptic peptide samples were analyzed via LC/MS/MS (*SI Appendix*, Fig. S3) at the Woods Hole Oceanographic Institution Fourier Transform Mass Spectrometry facility or in the Saito Lab using a Michrom Advance HPLC system coupled to a Thermo Scientific Q-Exactive Orbitrap mass spectrometer. Mass spectra were mapped to the translated whole genome of *T. oceanica* (NCBI Bioproject PRJNA36595) in Proteome Discoverer (version 2.2, Thermo). To identify putative homologs of Nox, *T. oceanica* GR, and reference flavoprotein disulfide reductases, BLASTP analysis was conducted online through the National Center for Biotechnology Information (NCBI), UniProt, and Ocean Gene Atlas. Full materials and methods are described in *SI Appendix, Experimental Methods*.

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